

# Probing the environment of neurotensin whilst bound to the neurotensin receptor by solid state NMR

P.T.F. Williamson<sup>a,c</sup>, S. Bains<sup>b</sup>, C. Chung<sup>b</sup>, R. Cooke<sup>b</sup>, A. Watts<sup>c,\*</sup>

<sup>a</sup>Laboratorium für Physikalische Chemie, ETH-Hönggerberg, CH-8093 Zurich, Switzerland

<sup>b</sup>GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK

<sup>c</sup>Biomembrane Structure Unit, Biochemistry Department, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Received 29 November 2001; revised 15 March 2002; accepted 19 March 2002

First published online 10 April 2002

Edited by Thomas L. James

**Abstract** A functionally active analogue of neurotensin, neurotensin(8–13), has been observed whilst bound to the agonist-binding site of the rat neurotensin receptor by nuclear magnetic resonance (NMR). Through the application of slow magic angle sample spinning and high-power proton decoupling, sufficient resolution and sensitivity were obtained in the carbon-13 spectrum to allow an assignment of many of the side chain resonances arising from uniformly carbon-13/nitrogen-15-labelled neurotensin(8–13) whilst bound to the neurotensin receptor. Significant perturbations in carbon-13 chemical shift were observed upon the binding of the neurotensin(8–13) to the receptor. Most importantly significant shifts were observed in both the carboxy terminus and tyrosine side chain of the neurotensin(8–13), suggesting that these sites are important in the interaction of the neurotensin with the agonist-binding site on the neurotensin receptor. Conversely, no perturbations were observed for the carbon-13 sites within the guanidinium groups of the arginine side chains, indicating little interaction with the receptor-binding site, or a shielding of the local environment by the surrounding nitrogen atoms. These NMR observations lend further support to previous structure–activity studies, site-directed mutagenesis and modelling studies of the agonist-binding site of the neurotensin receptor, from which the same specific residues for which NMR perturbations were observed are important for neurotensin receptor activation by neurotensin. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Neurotensin; Protein expression; Solid state; MAS; Nuclear magnetic resonance; G-protein coupled receptor

## 1. Introduction

The neurotensin receptor is a member of the G-protein coupled receptor (GPCR) family of transmembrane proteins, and is activated upon binding of the agonist neurotensin, a basic tridecapeptide, to the extracellular surface of cells. The neurotensin receptor is found widely in both the central and peripheral nervous system. In the periphery, the neurotensin receptor stimulates smooth muscle contraction [1] while in the central nervous system, the neurotensin receptor mediates a variety of activities including antinociception, hypothermia and increased locomotor activity [2–4]. These effects are prob-

ably mediated through the regulation of the mesolimbic and nigrostriatal dopamine pathways [5]. As a result, the pharmacological action of neurotensin is similar to that observed for dopamine, where compounds function as antipsychotics [6–8], and intervention may provide useful insights into the development of treatments for conditions such as schizophrenia [5] and Parkinson's disease [5].

To date no direct high-resolution structural information is available for the neurotensin receptor, possibly due to the limited success at producing 2D and 3D crystals for diffraction studies and also to the unfavourable relaxation rates associated with the size of membrane systems which hinder conventional high-resolution solution-state nuclear magnetic resonance (NMR) studies. Nevertheless, sequence analysis and modelling studies of the neurotensin receptor have suggested that the receptor adopts a seven transmembrane topology typical for the GPCR class of proteins [9]. Chimeric receptor studies [10] and site-directed mutagenesis analysis [9,11] have shown the location of the agonist-binding site on the receptor. These studies suggest that the third extracellular loop and the sixth transmembrane domain of the receptor are involved in the binding of the C-terminal domain of neurotensin. Solution-state NMR studies (NOEs and *J*-couplings) of the agonist, neurotensin, in the absence of receptor have revealed that no preferred conformation exists in solution [12]. Extensions of these studies to neurotensin in the presence of the membrane mimetic sodium dodecyl-d<sub>25</sub> sulphate, again confirm that no preferred conformation was adopted, although some ordering of charged residues on the surface of the micelles was observed [13]. In addition, extensive structure–function studies have been performed [14,15] indicating that residues 8–13 in the neurotensin are sufficient to elicit binding and activation of the neurotensin receptor.

The current study focusses on the application of NMR methodology to analyse the conformation and environment adopted by the neurotensin analogue, neurotensin(8–13), upon the binding to the detergent-solubilised neurotensin receptor. Typically, large detergent–receptor complexes produce poor resolution in the solution-state NMR spectrum due to a combination of residual anisotropic interactions caused by slow rotational diffusion, susceptibility broadening [16] and residual *B*<sub>0</sub> inhomogeneities. Through the application of slow magic angle sample spinning (500 Hz) and high-power proton decoupling (70 kHz), many of these anisotropic interactions were averaged sufficiently to allow the acquisition of high-resolution-like spectra, with sufficient intensity to permit the assignment of many of the side chain resonances for neu-

\*Corresponding author. Fax: (44)-1865-275268.

E-mail address: [awatts@bioch.ox.ac.uk](mailto:awatts@bioch.ox.ac.uk) (A. Watts).

rotensin(8–13) whilst bound to the agonist-binding site on the detergent-solubilised receptor. A detailed analysis of the perturbations in chemical shifts upon binding of neurotensin(8–13) to the receptor has allowed us to identify key sites that are important for the interaction of neurotensin with the receptor. Our results are interpreted in the context of current models proposed on the basis of site-directed mutagenesis and structure–activity studies.

## 2. Materials and methods

### 2.1. Solid phase synthesis of neurotensin(8–13)

Neurotensin(8–13) was synthesised using conventional Fmoc solid phase synthesis at the Oxford Centre for Molecular Sciences. Uniformly labelled amino acids (Promochem, UK) were protected and purified using standard amino acid protection protocols [17], and the nature and purity of the compounds was confirmed by electrospray mass spectroscopy and thin layer chromatography. Following solid phase synthesis, the peptide was purified by reverse phase high-performance liquid chromatography, eluting at an acetonitrile concentration of 27% comparable with standard neurotensin(8–13) (Sigma, UK). Electrospray mass spectroscopy of the final product gave a single molecular species with molecular weight of 868 Da, consistent with the uniform carbon-13 and nitrogen-15 labelling of neurotensin(8–13).

### 2.2. Expression and purification of the neurotensin receptor

Detergent-solubilised rat neurotensin receptor was obtained as described by Grisshammer [18–20]. For expression *Escherichia coli* strain DH5 $\alpha$  was grown on double strength TY medium [18–20] containing ampicillin (100 g/ml) and 0.2% glucose. A NTR fusion protein was expressed from the pRG/III-hs-MBPP-T43NTR-TrxA-H10 plasmid obtained from Dr R. Grisshammer [18–20]. This plasmid expresses a hybrid protein composed of a truncated neurotensin receptor with a maltose-binding protein, together with it periplasmic targeting sequence, attached to the N-terminus and thioredoxin and a deca-his tag to aid stability and purification to the C-terminus. The *E. coli* DH5 $\alpha$  were grown in 400 ml of medium in a 1 l flask at 37°C until OD<sub>600</sub> reached 0.7. The cultures were subsequently induced with 0.5 mM isopropyl- $\beta$ -galactoside. The temperature was then lowered to 20°C and further incubated for 40 h. The cells were harvested by centrifugation, flash frozen in liquid nitrogen and stored at –70°C.

The purification of heterologously expressed neurotensin receptor was performed as previously described by Grisshammer [18–20]. Briefly, 200 g of cell paste were resuspended in 1.2 l of neurotensin buffer (50 mM Tris, pH 7.4, 0.2 M NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM) containing pepstatin A, leupeptin A, phenylmethylsulfonyl fluoride, lysozyme and DNase. The cells were then broken using a flow through sonicator and the lysate clarified by centrifugation. The supernatant was then loaded onto a Qiagen NTA nickel affinity column in 1 mM imidazole at a flow rate of 10 ml min<sup>–1</sup>. The neurotensin receptor was then eluted in neurotensin

buffer containing 350 mM imidazole. The eluate was then concentrated using an Amicon stirred cell with YM-30 membrane.

The buffer was subsequently exchanged to a low-salt buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 20 mM NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM) using a 150 ml Sephadex-G25 column. The active receptor was then purified using a neurotensin affinity column [18,19]. The affinity column was equilibrated with low-salt buffer and the fraction containing the neurotensin receptor loaded at 0.5 ml min<sup>–1</sup>. Following washing with both low-salt buffer and 200 mM KCl buffer, the active neurotensin receptor was eluted using a high-salt buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1.0 M NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% LM). Prior to NMR studies the purified neurotensin receptor was returned to desalting buffer and concentrated using a stirred cell Amicon and Centricon containing an YM-30 membrane.

Prior to NMR measurements the concentration of protein was determined using an amido black protein assay. The specific activity of the sample was determined using a tritiated-neurotensin binding assay [18,20]. Typically the specific activity of the sample prior to NMR was 2.8 nmol mg<sup>–1</sup>. Although the theoretical activity based on the molecular weight of the protein is 10 nmol mg<sup>–1</sup>, such discrepancies between experimental and theoretical activities have been described previously and attributed to the buffer conditions employed for neurotensin binding assays [18,20]. The purity of the sample was determined by electrophoresis on a 5–12% gradient SDS–polyacrylamide gel followed by Coomassie staining. The gels show a single band consistent with the molecular weight of the expressed construct (data not shown). The NMR sample consisted of 10 nmol of neurotensin receptor in a 6 mm Chemagnetics rotor sealed to prevent dehydration during the course of the experiment. Neurotensin(8–13) was added sequentially until a stoichiometric quantity had been added, ensuring that after each addition only a single population of neurotensin(8–13) was present. Neurotensin(8–13) was added as a concentrated solution in buffer, such that no more than 15  $\mu$ l of neurotensin(8–13) was added to each sample.

### 2.3. NMR methodology

All carbon-13 spectra were acquired at 125.7985 MHz on Chemagnetics CMX-500, with triple resonance 6 mm CP-MAS probe. All carbon-13 spectra were acquired using standard proton-decoupled carbon-13 acquisition, with a spinning frequency of 500 Hz. Carbon-13 pulse lengths were typically 5  $\mu$ s and 70 kHz proton decoupling was applied during acquisition. All spectra were acquired at 5°C unless otherwise stated.

## 3. Results and discussion

### 3.1. Assignment

Assignment of neurotensin(8–13) was performed in low-salt buffer used for the final preparation of the neurotensin receptor prior to binding studies and NMR experiments. The 1D carbon-13 MAS spectra of neurotensin(8–13) is shown in Fig. 1. The data obtained under these conditions show good

Table 1

Residue	Carbon-13 chemical shift (ppm)					
	CO	C $\alpha$	C $\beta$	C $\gamma$	C $\delta$	Other
Arg <sup>1</sup>	169.43 (+1.51)	52.08	28.27	24.22	40.39	156.40 (C $\epsilon$ )
Arg <sup>2</sup>	171.24	50.86	27.52	24.22	40.39	156.40 (C $\epsilon$ )
Pro <sup>3</sup>	172.72	59.77	28.30	23.64	47.86	
Tyr <sup>4</sup>	172.05	54.18	35.70			127.26 (–0.13) (C1) 130.44 (–0.62) (C2,6) 115.14 (–0.15) (C3,5) 154.20 (+0.2) (C4)
Ile <sup>5</sup>	171.76	57.38	35.59	14.34 (+0.11) 23.16	9.69 (+0.07)	
Leu <sup>6</sup>	178.05 (+0.43)	52.78	40.39	24.22	20.80 (+0.27) 22.02	

Assignment of neurotensin(8–13) in buffer as described in text and the perturbations of resolvable resonances upon binding to the neurotensin receptor are given in italics. All chemical shifts referenced externally to adamantane whose low field peak is given as 37.6 ppm. For the determination of perturbations in chemical shift, referencing was confirmed from resonances arising from the buffer components.

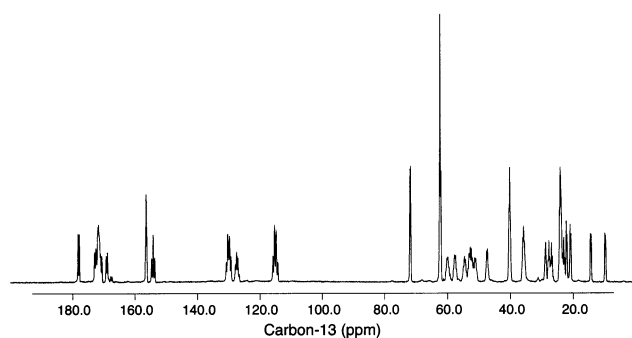


Fig. 1. Proton-decoupled carbon-13 MAS spectra of 4 mg uniformly carbon-13/nitrogen-15-labelled neurotensin(8–13) in low-salt buffer. Data acquired at 5°C and averaged over 1024 acquisitions. Data zero filled to 32768 points and processed with 3 Hz line broadening prior to Fourier transform.

resolution (linewidths typically 10 Hz), with many of the carbon–carbon  $J$ -couplings well resolved. With the exception of the two resonances arising from the natural abundance of glycerol in the sample ( $\delta = 62.2$  ppm and  $\delta = 71.79$  ppm), all other resonances can be assigned to the uniformly labelled neurotensin(8–13). On the basis of the distinct chemical shifts and the observed carbon–carbon and carbon–nitrogen  $J$ -couplings, many of the side chain resonances can be readily assigned from the proton-decoupled carbon-13 MAS spectra and by comparison with previously published chemical shifts on model peptide systems [21]. Assignment of other resonances was performed on the basis of a 2D-MAS-COSY spectrum (data not shown) of uniformly carbon-13/nitrogen-15-labelled neurotensin(8–13) where the backbone  $C\alpha/CO$  resonances were well resolved and could be assigned on the basis of connectivities with side chain resonances. These assignments are given in Table 1.

### 3.2. Binding studies

The proton-decoupled spectra of neurotensin receptor in detergent micelles is shown in Fig. 2A. The spectrum is dominated by two intense resonances at 62 and 71 ppm arising from the natural abundance carbon-13 within the glycerol within the sample. Additional intensity is also apparent in the region between 10 and 80 ppm which has been shown to arise from natural abundance carbon-13 present in the CHAPS, dodecyl maltoside and cholesterol hemisuccinate present in the buffer system. Resonances downfield from these have been assigned to natural abundance carbons in the cholesterol hemisuccinate rings and from the carbonyl groups present in several of the detergent components. From the spectra presented, no resonances were observed from that natural abundance carbon-13 present from the detergent-solubilised receptor. We attribute this to the low concentration of protein present in the sample (10 nmol) with carbon-13 present at only natural abundance levels.

The spectra of detergent-solubilised neurotensin receptor upon the addition of a stoichiometric amount of uniformly labelled neurotensin(8–13) is shown in Fig. 2B. The spectrum is again dominated by intense resonances arising from natural abundance material within the sample. However, upon the addition of 10 nmol of neurotensin(8–13), several resonances clearly appear in the downfield region of the spectra (110–185 ppm) (Fig. 3). We attribute these resonances to well-resolved sites within the side chains of aromatic and basic residues

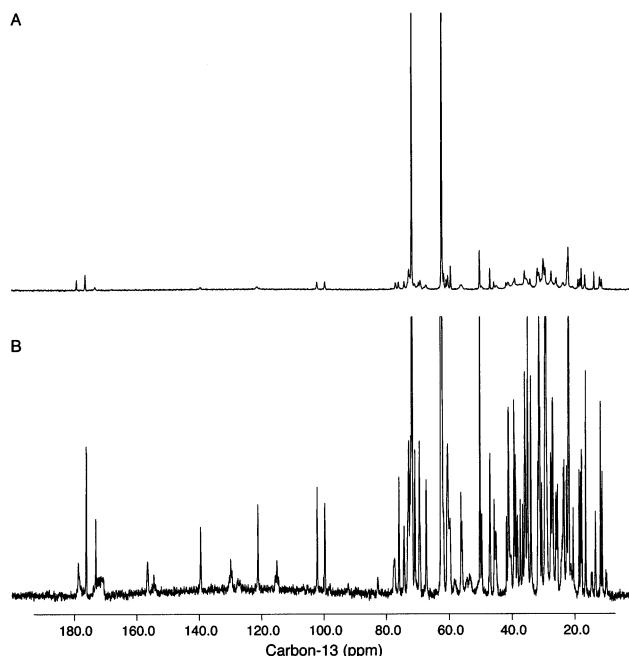


Fig. 2. Proton-decoupled carbon-13 MAS spectra of 10 nmol of neurotensin receptor in low-salt buffer (A) and upon the addition of 10 nmol of uniformly carbon-13/nitrogen-15-labelled neurotensin(8–13) ( $\times 10$ ) (B). Data acquired at 5°C and averaged over 8192 acquisitions and processed with 3 Hz line broadening prior to Fourier transform.

present in the neurotensin(8–13) on the basis of the similarity of the chemical shifts with those that have been described for the neurotensin(8–13) under identical buffer condition. In addition to these well-resolved resonances present in the downfield region, careful analysis of the aliphatic region allows the assignment of several of resonances arising from aliphatic resonances in the leucine and isoleucine side chains. Analyses of the carbonyl region also shows increased intensity in addition to that arising from the carbonyl groups from the detergents present, and in several cases carbonyl shifts that were

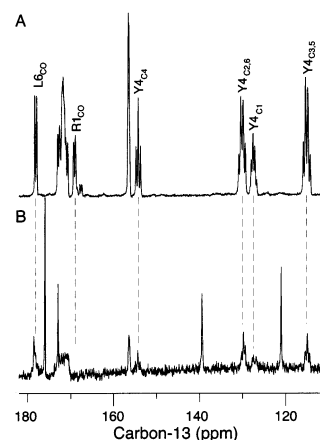


Fig. 3. Expansion of carbon-13 MAS spectrum from 110 to 185 ppm of neurotensin(8–13) (A) and neurotensin(8–13) bound to the detergent-solubilised receptor (B). The spectral region shown contains resonances arising from carbonyl groups and some sites within the arginine and tyrosine side chains. The assigned sites represent those for which perturbations were observed upon binding of the ligand to receptor.

clearly assignable are no longer present. The perturbations of these chemical shifts from the values obtained in detergent buffer alone are given in Table 1 (in italics) and for the region between 110 and 185 ppm indicated in Fig. 3. Although many of the side chain resonances are well resolved, many of the backbone  $C_{\alpha}$  aliphatic side chains are masked by the presence of the strong natural abundance from the detergent.

Although many of the perturbations in chemical shift are relatively small, the good resolution obtained in the system (line widths typically less than 10 Hz), in addition to the presence of natural abundance peaks that show no perturbation between the two samples, suggest that the perturbations observed arise solely from the interaction with the neurotensin receptor present in the detergent micelles. We preclude the possibility of non-specific binding on the basis of tritiated binding assays that show that non-specific binding within the system is less than 5% of the bound ligands under these conditions [18,20]. Additionally, the presence of a single distinct resonance for sites where perturbations have been observed indicates strongly that the neurotensin(8–13) present in the sample exists in a single, predominantly bound form.

Many of the resolved resonances for labelled neurotensin (8–13) show either no or only small perturbations in chemical shift upon binding. However, significant changes were observed for two of the previously well-resolved carbonyl shifts, and the tyrosine side chain (Fig. 3). In addition, more subtle changes were observed for the resonances arising from the aliphatic side chains present in the neurotensin(8–13). Carbon-13 chemical shifts are sensitive to a variety of environmental changes including molecular conformation, local charged environment and perturbation of the local field due to the presence of ring current effects arising from the presence of aromatic groups in the vicinity ( $< 4$  Å) of the labelled site [22–24]. The perturbations in chemical shifts observed can be interpreted in terms of molecular rearrangements and changes in local electrostatic environment upon the binding of neurotensin to the receptor compared with the receptor in free solution.

The best characterised carbon-13 chemical shifts are those arising from protein backbone resonances [25,26]. Although many of these resonances are poorly resolved, both the carbonyl groups from Arg<sup>1</sup> and the carboxy terminus were clearly resolved in the carbon-13 MAS spectra of neurotensin in detergent buffer. Upon binding, the resonance from the carbonyl group of Arg<sup>1</sup> moves downfield by 1.51 ppm, such downfield changes in carbonyl chemical shifts in more extended secondary structural motifs are indicative of the local region adopting a more helical conformation ( $\psi \approx 40^\circ$ ) [25,26]. However, the absence of assignments for others of the carbonyl and  $C_{\alpha}$  resonance due to poor site resolution precludes a more detailed analysis of the backbone conformation. The relatively high chemical shift of the carboxy terminus in both the unligated and receptor-bound forms of the neurotensin(8–13) might suggest that the peptide exists in its deprotonated state both in solution and when bound. The further downfield shift upon binding suggests that this group might be interacting with other charged sites within the ligand-binding site. Such an analysis is consistent with structure–activity relationship studies which show a two-fold reduction in affinity for the receptor for both neurotensin and neurotensin(8–13) upon the conversion of the carboxylic group to an amide [14]. This observation would also be in agreement

with the site-directed mutagenesis and modelling studies performed by Barroso et al. [11], which suggest the existence a strong ionic interaction between the carboxy terminus and an arginine in the agonist-binding site.

Perturbations in the side chain chemical shifts were observed for both of the aliphatic amino acids and tyrosine. The small perturbations in chemical shift in the aliphatic side chains suggest that large changes in electrostatic environment due to changes in conformation or local environment are observed upon the binding of the ligand to the receptors do not occur. This observation is consistent with the view that these residues weakly interact with the agonist-binding site through hydrophobic interactions [9,11].

Larger perturbations in resonances assigned to the tyrosine side chain were observed upon the binding of neurotensin(8–13) to the receptor. Previous studies have shown that the C1 carbon in the aromatic ring is sensitive to the orientation of the ring with respect to the backbone [27], and thus perturbations of this resonance might be indicative of a change in conformation of the side chain upon binding. However, the information presented here cannot preclude the observed perturbation arising from changes in local environment. The perturbations observed in the C2,6, C3,5 and C4 resonances strongly suggest that the tyrosine is interacting with sites within the receptor-binding pocket. Structure–activity studies of neurotensin and neurotensin(8–13) suggest that the tyrosine contains vital pharmacophores necessary for binding to the receptor [14,15] and suggest that the tyrosine side chain may be involved in both  $\pi$ – $\pi$  stacking and hydrogen bonding with either receptor or ligand upon receptor binding. These observations have gained further support from both mutagenesis and modelling studies [9,11]. The data presented here does not allow us to deconvolute the possible contributions of such interactions, but further supports the importance of this group in the binding and activation of the neurotensin receptor by neurotensin(8–13) and neurotensin.

Resonances arising from the arginine- $C_{\xi}$  are also clearly resolved in the carbon-13 MAS spectra of neurotensin(8–13) whilst bound to the neurotensin receptor in detergent micelles. The absence of significant perturbations in chemical shift suggests the  $C_{\xi}$ s have undergone few detectable changes in electrostatic environment or conformation upon binding to the receptor. Structure–activity studies suggest that the role of Arg<sup>8</sup> in the binding of neurotensin may be minimal with relatively small changes in affinity upon its modification [14,15]. These views are supported by several modelling studies which suggest that the guanidinium group of Arg<sup>8</sup> is only interacting weakly with the receptor [9,11], indicating that few perturbations in chemical shift are expected. In contrast, site-directed structure–activity and mutagenesis studies have shown that the guanidinium group of Arg<sup>9</sup> plays an important role in the binding of neurotensin (and neurotensin(8–13)) to the receptor [9,11]. The absence of any detectable change in either of the two arginine- $C_{\xi}$  resonances would suggest that the proposed interactions between the guanidinium group and aromatic systems within the binding site are weak, with neither of the guanidinium groups approaching aromatic systems closer than (4–5 Å). This might be due to the central location of the carbon atom in the centre of the guanidinium group which would preclude close contacts, in agreement with the ligand geometries proposed by Pang et al. [9] on the basis of modelling and site-directed mutagenesis studies.



#### 4. Conclusions

The above results indicate that sufficient resolution in the carbon-13 NMR spectra is attainable through the application of slow magic angle sample spinning and high-power proton decoupling to permit a detailed analysis of the chemical shifts of neurotensin(8–13) whilst bound to detergent-solubilised receptor. Although many of the backbone and aliphatic resonances arising from carbon-13 sites within the neurotensin(8–13) were obscured by strong resonances arising from natural abundance carbon-13 within the buffer system, many of the sites from aromatic and basic side chains were clearly resolvable. The perturbations in chemical shift observed upon the binding of the neurotensin(8–13) to the detergent-solubilised receptor indicate key residues that are important in the binding of the neurotensin to the receptor. The magnitude of the perturbations observed for both the tyrosine side chain and the carboxy terminus indicate clearly that these sites interact strongly with the receptor upon binding. These direct observations are in good agreement with observations from site-directed mutagenesis, structure–function and modelling studies of neurotensin(8–13) interacting with the neurotensin receptor.

**Acknowledgements:** Maureen Petkeathly at the Oxford Centre for Molecular Sciences is acknowledged for the solid phase synthesis of neurotensin(8–13). Gerhard Gröbner at the University of Umeå and Paul Spooner and Rene Verel at the University of Oxford are gratefully acknowledged for their valuable discussions during the course of this work.

#### References

- [1] Kitabgi, P. (1982) *Ann. N. Y. Acad. Sci.* 400, 37–55.
- [2] Kachur, J.F., Miller, R.J., Field, M. and Rivier, J. (1982) *J. Pharmacol. Exp. Ther.* 1982, 456–483.
- [3] Ervin, G.N., Birrema, L.S., Nemeroff, C.B. and Prange, A.J. (1981) *Nature* 291, 73–76.
- [4] Osbahr, A.H., Nemeroff, C.B., Manberg, P.J. and Prange, A.J. (1979) *Eur. J. Pharmacol.* 54, 299–302.
- [5] Uhl, G.R., Whitehouse, P.J., Price, D.L., Tourtelotte, W.W. and Kuhar, M.J. (1984) *Brain Res.* 308, 186–190.
- [6] Garcia-Sevilla, J.A., Magnusson, T., Carlsson, J., Leban, J. and Folkers, K. (1978) *Arch. Pharmacol.* 305, 213–218.
- [7] Widerlov, E., Kilts, C.D., Mailman, R.B., Nemeroff, C.B., Prange, A.J. and Bresse, G.R. (1982) *J. Pharmacol. Exp. Ther.* 224, 1–6.
- [8] Reches, A., Burke, R.E., Jiang, D., Wagner, H.R. and Fahn, S. (1983) *Peptides* 4, 43–48.
- [9] Pang, Y.P., Cusack, B., Groshan, K. and Richelson, E. (1996) *J. Biol. Chem.* 271, 15060–15068.
- [10] Cusack, B., Groshan, K., McCormick, D.J., Pang, Y.P., Perry, R., Phung, C.T., Souder, T. and Richelson, E. (1996) *J. Biol. Chem.* 271, 15054–15059.
- [11] Barroso, S., Richard, F., Nicolas-Etheve, D., Reversat, J.L., Bernassau, J.M., Kitabgi, P. and Labbe-Julie, C. (2000) *J. Biol. Chem.* 275, 328–336.
- [12] Nieto, J.L., Rico, M., Santoro, J., Herranz, J. and Bermejo, F.J. (1986) *Int. J. Pept. Protein Res.* 28, 315–323.
- [13] Xu, G. and Deber, C.M. (1991) *Int. J. Pept. Protein Res.* 37, 528–535.
- [14] Seffler, A.M. et al. (1995) *J. Med. Chem.* 38, 249–257.
- [15] St-Pierre, S., Lalonde, J.M., Gendreau, M., Quirion, R., Regoli, D. and Rioux, F. (1981) *J. Med. Chem.* 24, 370–376.
- [16] Swiet, T.M., Tomaselli, M., Huerlimann, M.D. and Pines, A. (1998) *J. Mag. Res.* 133, 386–387.
- [17] Jones, J. (1992) in: *Oxford Chemistry Primer*, Oxford University Press, Oxford.
- [18] Tucker, J. and Grisshammer, R. (1996) *Biochem. J.* 317, 891–899.
- [19] Grisshammer, R., Averbeck, P. and Sohal, A.K. (1999) *Biochem. Soc. Trans.* 27, 899–903.
- [20] Grisshammer, R. and Tucker, J. (1997) *Protein Expr. Purif.* 11, 53–60.
- [21] Wuthrich, K. (1986) *NMR of Protein and Nucleic Acids*, John Wiley and Sons, New York.
- [22] Asakura, T., Taoka, K., Demura, M. and Williamson, M.P. (1995) *J. Biomol. NMR* 6, 227–236.
- [23] Williamson, M.P. and Asakura, T. (1993) *J. Mag. Res. Ser. B* 101, 63–71.
- [24] Williamson, P.T.F., Grobner, G., Spooner, P.J.R., Miller, K.W. and Watts, A. (1998) *Biochemistry* 37, 10854–10859.
- [25] Wishart, D.S., Sykes, B.D. and Richards, F.M. (1992) *Biochemistry* 31, 1647–1651.
- [26] Wishart, D.S. and Sykes, B.D. (1994) *J. Biomol. NMR* 4, 171–180.
- [27] Miyamoto, H., Komoto, T., Kurosu, H., Ando, I., Ozaki, T. and Shoji, A. (1990) *J. Mol. Struct.* 220, 251–260.